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Lab Resource: Multiple Cell Lines

Generation of human induced pluripotent stem cell lines (UNIMGi003-A and UNIMGi004-A) from two Italian siblings affected by Unverricht-Lundborg disease

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A B S T R A C T

Unverricht-Lundborg disease (ULD) is an inherited form of progressive myoclonus epilepsy caused by mutations in the gene encoding Cystatin B (CSTB), an inhibitor of lysosomal proteases. The most common mutation described in ULD patients is an unstable expansion of a dodecamer sequence located in the CSTB gene promoter. This expansion is causative of the downregulation of CSTB gene expression and, consequently, of its inhibitory activity. Here we report the generation of induced pluripotent stem cell (iPSC) lines from two Italian siblings having a family history of ULD and affected by different clinical and pathological phenotypes of the disease.

1. Resource table

Unique stem cell lines identifier	UNIMGi003-A UNIMGi004-A
Alternative name(s) of stem cell lines	ULD1 ULD2
Institution	University "Magna Græcia" of Catanzaro
Contact information of distributor	Valeria Lucchino
Type of cell lines	iPSCs
Origin	Human
Additional origin info required for human iPSC	UNIMGi003-A (ULD1): Age: 53 Sex: Male Ethnicity: Caucasian UNIMGi004-A (ULD2): Age: 48 Sex: Female Ethnicity: Caucasian
Cell Source	Blood T-lymphocytes (ULD1); Skin fibroblasts (ULD2)
Clonality	Mixed
Associated disease	Unverricht-Lundborg disease
Gene/locus	Cystatin B (EPM1) gene
Date archived/stock date	09/02/2021
Cell line repository/bank	https://hpscreg.eu/cell-line/UNIMGi003-A https://hpscreg.eu/cell-line/UNIMGi004-A
Ethical approval	The human study was approved by the Ethics Committee of the *"Magna Graecia" University of Catanzaro* and the *Azienda Ospedaliero – Universitaria "Mater Domini"* (Approval number: AOM102_2020) with written informed consent obtained from each participant

2. Resource utility

The generated iPSC lines represent a useful tool for the establishment of an *in vitro* model of Unverricht-Lundborg disease. Neuronal cells differentiated from these iPSC lines will provide the opportunity to identify the molecular mechanisms responsible for the disease and to establish high-throughput drug screenings directly on the patient's cells.

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal (Human embryonic stem cell-like morphology)	Fig. 1 panel A
Phenotype	Qualitative analysis – Immunocytochemistry	Positive staining for pluripotency markers: Oct4, Nanog, TRA-1–60	Fig. 1 panel B
	Quantitative analysis – qRT-PCR – PluriTest	All pluripotency genes tested presented a fold change (FC) expression of at least 165 relative to parental fibroblasts. SCORE PLURITEST: ULD1: novelty = 1,56 Pluripotency = 18,72. ULD2: novelty = 1,47 Pluripotency = 20,38.	Fig. 1 panel C (qRT-PCR) and D (PluriTest)
Genotype	Karyotype (G-banding) and resolution	46, XY for ULD1 line; 46, XX for ULD2 line. 500-band resolution; magnification 100X.	Fig. 1 panel E
Identity	Microsatellite	N/A	N/A
	STR analysis	Genetic characteristics were determined by PCR-single-locus-technology. 16 independent PCR-systems D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, AMEL, D5S818, FGA, D19S433, vWA, TPOX and D18S51 were investigated All STR markers matched between the generated iPSC lines and their corresponding parental cell line (fibroblasts/T-lymphocytes)	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	N/A	N/A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR (Abm Mycoplasma PCR Detection Kit (Cat. # G238)): negative	Not shown but available with author
Differentiation potential	Embryoid body formation	Immunofluorescence assay of differentiated EBs for Brachyury (mesoderm), Nestin (ectoderm), and Sox17 (endoderm)	Fig. 1 panel F
List of recommended germ layer markers	Expression of these markers has to be demonstrated at mRNA (RT PCR) or protein (IF) levels, at least 2 markers need to be shown per germ layer	Ectoderm: PAX6 Endoderm: FOXA2 Mesoderm: HAND1	qRT-PCR with reference genes (Suppl. Fig. 1B)
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

3. Resource details

Unverricht-Lundborg disease (ULD), also known as progressive myoclonic epilepsy-1A (EPM-1A), is an autosomal recessively inherited neurodegenerative disorder characterized by onset at age 6–16 years, action- and stimulus-sensitive myoclonus, generalized epilepsy, tonic clonic seizures with dementia and ataxia, but only a mild mental deterioration without formation of inclusion bodies in the brain. The main gene underlying ULD, *CSTB*, was identified in 1996 (Pennacchio et al., 1996) and encodes Cystatin B, an inhibitor of cysteine proteases, including those of the cathepsins family. The most common mutation described in ULD pathogenesis is the unstable expansion (>30 times) of a tandem repeat of a dodecamer (5′-CCCCGCCCCGCCG-3′) located in the 5′ flanking region of the *CSTB* promoter, 175 bp upstream from the translation initiation codon (Lalioti et al., 1997). This expansion mutation is mostly detected in homozygous form, but it can occur also in compound heterozygous form with one of the rarer coding-region missense and stop mutations (Joensuu et al., 2007). The mutation leads to a downregulation of *CSTB* gene expression and reduction of Cstb protein expression and reduced inhibitory activity (Rinne et al., 2002). Patients of this study are two Italian siblings, named ULD1 and ULD2, affected by different phenotypic and clinical degrees of ULD disease, with a severe and a mild form of the disease, respectively. ULD1 and ULD2 belong to a clinically well-characterized Mediterranean myoclonus family (Lehesjoki et al., 1994) (FAM. 6). The age of disease onset was 12 years for ULD1 and 11 years for ULD2 and tonic-clonic seizures were the first sign of the disease. T-lymphocytes isolated from ULD1 patient and skin fibroblasts isolated from ULD2 patient were reprogrammed to iPSCs via Sendai virus (SeV) carrying the four reprogramming factors *OCT4, SOX2, KLF4*, and *c-MYC* (Figure Supplementary Fig. 1A, Table 1). The resulting iPSC colonies exhibited typical human embryonic stem cell (hESC)-like morphology, with tightly packed cells and sharp edges (Fig. 1A, Table 1). The loss of SeV-derived transgenes was confirmed by qRT-PCR (Figure Supplementary Fig. 1A, Table 1. TL = T-lymphocytes; iTL = infected T-lymphocytes; SF = skin fibroblasts; iSF = infected skin fibroblasts). Immunostaining was performed to assess the positive expression of the following pluripotency markers: Oct4, Nanog, and TRA-1–60 (Fig. 1B, Table 1), while the expression of endogenous pluripotency-associated genes (*OCT4*, *SOX2*, *REX1*, and *NANOG*) was evaluated by qRT-PCR (Fig. 1C, Table 1). Pluripotency was also confirmed with the PluriTest-based genome-wide gene expression profile (Fig. 1D, Table 1). Conventional cytogenic analysis on ULD1- (46, XY) and ULD2- (46, XX) iPSC lines showed normal karyotype without chromosomal abnormalities (Fig. 1E, Table 1). Furthermore, iPSCs-derived embryoid bodies (EBs) (Figure Supplementary Fig. 1C) were able to differentiate towards the three germ layers, as confirmed by qRT-PCR (Figure Supplementary Fig. 1B, Table 1) and immunostaining (Fig. 1F, Table 1) of markers associated to mesoderm, ectoderm, and endoderm.

4. Materials and methods

4.1. Reprogramming and iPSC culture

T-lymphocytes isolated from peripheral blood mononuclear cells (PBMCs) were cultured on 10 μg/mL CD3-coated dishes with AIM-V medium supplemented with 20% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, and 125 ng/mL Interleukine-2 (IL-2). Skin fibroblasts were isolated and expanded by the outgrowth method in DMEM medium containing 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. Reprogramming to pluripotency was carried out by non-integrating Sendai-virus (SeV)-mediated (CytoTune™-iPS 2.0 Sendai

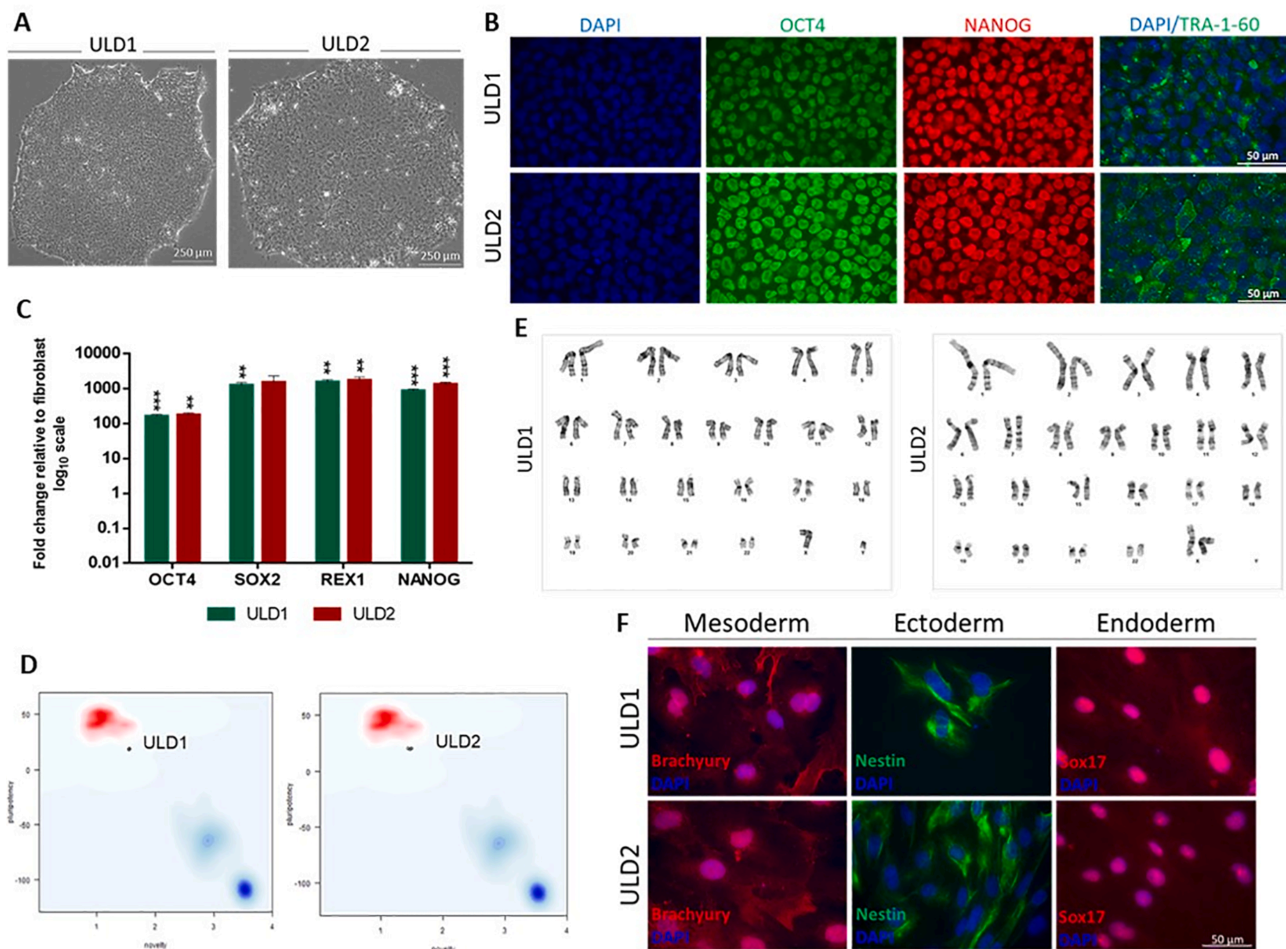

Fig. 1.

Reprogramming Kit, Thermo Fisher Scientific) transfection of the four canonical transcription factors (*OCT4*, SOX2, *KLF4* and *c-MYC*) of pluripotency. Emerged iPSCs clones were manually picked and cultured on Matrigel (BD Biosciences)-coated dishes in mTeSR1 medium (STEMCELL Technologies), in a humidified incubator at 37 °C at 5% CO_2. All cell lines were tested for Mycoplasma before being used in experiments.

4.2. RNA isolation, RT-PCR, and qRT-PCR

Total RNA was extracted using TRIzol reagent and reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). 500 ng of cDNA were amplified by standard PCR to perform SeV detection, while 22 ng of cDNA were amplified by qRT-PCR using Power SYBR Green Master Mix (Applied Biosystems) for gene expression quantification. The *GAPDH* housekeeping gene was used for internal control. A list of primers is provided in Table 2.

4.3. Embryoid body formation assay

Single-cell suspensions of iPSC colonies were seeded on low attachment (poly-2-hydroxyethyl methacrylate-coated) dishes and cultured for three days in mTeSR1 medium supplemented with 10 μM Rho-kinase inhibitor (Y-27632, Selleckchem) to obtain cell aggregation. At day 7, newly formed EBs were transferred on 5 μg/ml Biolaminin 521LN-coated dishes and cultured in DMEM-F12 medium supplemented with 20% knockout serum replacement (KSR, Thermo Fisher Scientific), 1% Glutamax, 1% NonEssential Amino Acids, 100 μM 2-mercaptoethanol, and 0.5% penicillin and streptomycin for 10 days before collecting for immunofluorescence.

4.4. Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 10 min at RT, washed in DPBS and blocked for 30 min at RT with blocking solution (BS) (PBS + 1% BSA + 0,1% Triton-X-100), followed by overnight incubation at +4 °C with primary antibodies (listed in Table 2) diluted in BS. The next day, the cells were washed with DPBS and incubated with secondary antibodies (listed in Table 2) for 30 min at RT. Nuclei were stained with DAPI and the slides were mounted with Dako Fluorescent mounting medium. Images were acquired with imaging systems (DMi8), filter cubes and software from Leica Microsystems.

4.5. Karyotype analysis

Karyotyping analysis was performed following standard cytogenetic procedures at the Medical Genetics Unit of Mater Domini University Hospital (Catanzaro, Italy). Briefly, cells were treated with 0,1 μg/mL Nocodazole solution (ACROS Organics) for 4 h at 37 °C and then detached and washed in DPBS before treatment with hypotonic solution (0,033 M KCl and 0,017 M $H_3CCOONa$) for 20 min at 37 °C, followed by fixation in methanol/acetic acid solution (3:1 v/v). Giemsa (G)-based chromosomal banding was performed on fixed metaphases according to conventional protocols. DMRA fluorescent microscope (Leica Microsystems, Germany) was used for image acquisition of banded chromosomes and CytoVision® 7.3.1 software (Leica Biosystems, Germany) for analysis performed on 20 metaphases.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Mouse anti-OCT4	1:100	STEMCELL Technologies, Cat# 60,093	RRID: AB_2801346
Pluripotency Markers	Rabbit anti-NANOG	1:200	Thermo Fisher Scientific, Cat# PA1-097	RRID: AB_2539867
Pluripotency Markers	Mouse anti-TRA-1–60	1:100	Thermo Fisher Scientific, Cat# 41-1000	RRID: AB_2533494
Differentiation Markers	Mouse anti-NESTIN	1:1000	STEMCELL Technologies, Cat# 60091AD	RRID: AB_2650581
Differentiation Markers	Goat anti-BRACHYURY	1:20	R&D Systems, Cat# AF2085	RRID: AB_2200235
Differentiation Markers	Goat anti-SOX17	1:20	R&D Systems, Cat# AF1924	RRID: AB_355060
Secondary antibodies	Alexa Fluor® 647 Goat anti-mouse IgG	1:500	Thermo Fisher Scientific, Cat# A-21235	RRID: AB_2535804
Secondary antibodies	Alexa Fluor® 488 Goat anti-rabbit IgG	1:500	Thermo Fisher Scientific, Cat# A-11008	RRID: AB_143165
Secondary antibodies	Alexa Fluor® 594 Donkey anti-goat IgG	1:500	Thermo Fisher Scientific, Cat# A-11058	RRID: AB_2534105

Primers			
	Target	Size of band	Forward/Reverse primer (5′–3′)
Sendai virus (PCR)	SeV	181 bp	GGATCACTAGGTGATATCGAGC / ACCAGACAAGAGTTTAAGAGATATGTATC
Transgene	c-MYC	532 bp	TAACTGACTAGCAGGCTTGTCG / TCCACATACAGTCCTGGATGATGATG
Transgenes	Polycistronic KOS (KLF4, OCT3/4, SOX2)	528 bp	ATGCACCGCTACGACGTGAGCGC / ACCTTGACAATCCTGATGTGG
Pluripotency Markers (qPCR)	OCT4	64 bp	GGAGGAAGCTGACAACAATGAA / GGCCTGCACGAGGGTTT
Pluripotency Markers (qPCR)	SOX2	151 bp	GGGAAATGGGAGGGGTGCAAAAGAGG / TTGCGTGAGTGTGGATGGGATTGGTG
Pluripotency Markers (qPCR)	NANOG	116 bp	TGCAAGAACTCTCCAACATCCT / ATTGCTATTCTTCGGCCAGTT
Pluripotency Markers (qPCR)	REX1	190 bp	GTGTGAACAGAACAGAAGAGGC / CTGGTGTCTTGTCTTTGCCC
House-Keeping Genes (qPCR)	GAPDH	167 bp	TCCTCTGACTTCAACAGCGA / GGGTCTTACTCCTTGGAGGC
Germ layer markers (qPCR)	PAX6	112 bp	CAGCTTCACCATGGCAAATAA / ATCATAACTCCGCCCATTCA
Germ layer markers (qPCR)	FOXA2	187 bp	TGAAGATGGAAGGGCACGAG / GACGACATGTTCATGGAGCC
Germ layer markers (qPCR)	HAND1	190 bp	CCAGCTACATCGCCTACCTG / CCGGTGCGTCCTTTAATCCT

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102329.

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